



## Research article

Prebiotic activity of paramylon isolated from heterotrophically grown *Euglena gracilis*Tanmay Bhattad<sup>a</sup>, Akshaykumar Koradiya<sup>b</sup>, Gunjan Prakash<sup>a,\*</sup><sup>a</sup> DBT-ICT Centre for Energy Biosciences, India<sup>b</sup> Department of Food Engineering and Technology, Institute of Chemical Technology, Mumbai, India

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## ABSTRACT

Paramylon from *Euglena gracilis* is an insoluble crystalline  $\beta$ -1,3-glucan which have pharmaceutical and nutraceuticals applications. The present study aims to check the prebiotic potential of paramylon derived from heterotrophically grown *E. gracilis* in bioreactor. The Paramylon was extracted using sodium dodecyl sulfate from *E. gracilis* biomass. The Fourier Transform-Infra Red spectroscopy and scanning electron microscopy demonstrated the isolated paramylon to be equivalent to that of analytical standard. The prebiotic activity of *E. gracilis* cell extract and isolated paramylon was studied. *E. gracilis* cell extract as well as isolated paramylon led to cell number enhancement of Lactid (*Lactobacillus*) strain exhibiting the prebiotic activities.

## 1. Introduction

Microalgae have conventionally been known for their nutritional properties. *Euglena gracilis* is a freshwater unicellular flagellate, photosynthetic prokaryote and eukaryotic microalgae that can grow in a wide range of environment, and has multiple biotechnological applications (Mahapatra et al., 2013; Gissibl et al., 2019). *E. gracilis* produces high-value products that include all 20 amino acids, polyunsaturated long-chain fatty acids, vitamins, minerals and  $\beta$ -1,3-glucan known as paramylon (Gissibl et al., 2019). The global market for  $\beta$ -1,3-glucan is projected to reach US\$867.7 million by the year 2027 with a compound annual growth rate of 7 % (<https://www.globenewswire.com/news-release/2020/08/14/2078387/0/en/Global-Beta-Glucan-Industry.html>). There are multiple plant and microbial sources for  $\beta$ -1,3-glucan like cell wall cereal grains, mushroom and baker's yeast (Zhu et al., 2016). Baker's yeast i.e. *Saccharomyces cerevisiae* is one of the main commercial source for the  $\beta$ -1,3-glucan production. The  $\beta$ -glucan content in yeast has been found to be less than 15% of its dry weight and is intracellular which makes its isolation an energy intensive process due to presence of tough cell wall (Zhu et al., 2016). *E. gracilis* can accumulate large quantities of  $\beta$ -1,3-glucan in the range of 20–75% of dry weight when cultivated in the presence of adequate carbon sources (Muchut et al., 2018). *E. gracilis* is devoid of cell wall and therefore  $\beta$ -1,3-glucan extraction is much easier as compared to that of *S. cerevisiae*. Moreover, paramylon is a linear  $\beta$ -glucan which comprises of only  $\beta$ -1,3 linkage that has been found to activate macrophages and human

lymphocytes and to have immunomodulatory effects (Kataoka et al., 2002; Bohn and BeMiller, 1995).  $\beta$ -glucan is insoluble in aqueous/lipophilic system and cannot be digested by humans (due to lack of  $\beta$ -1,3 glucanase in the human digestive system). This property classifies them as prebiotic dietary fibre (Snart et al., 2006; Lam and Cheung, 2013). Therefore,  $\beta$ -1,3-glucan from *E. gracilis* can be an effective alternative to replace yeast derived  $\beta$ -1,3/1,6-glucan pharmaceutical and nutraceuticals sector. The potential of paramylon in pharmaceutical sector is well established as immunomodulator (Barsanti and Gualtieri, 2019). However, there is no specific study to establish paramylon as functional dietary fibre and to demonstrate its prebiotic potential on the growth of probiotic bacteria. In the present study, the paramylon was isolated from the heterotrophically grown cells. The functional characterization of *E. gracilis* as whole cell and isolated paramylon was executed for their prebiotic potential and compared with *Chlamydomonas reinhardtii* (a non-paramylon producing algae). The study establishes the proof of concept for the whole *Euglena* cells and paramylon to have prebiotic activities.

## 2. Materials and methods

## 2.1. Microalgae culture conditions and cultivation

*E. gracilis* Klebs strain (NIES-48) was procured from National Institute for Environmental Studies of Japan and was cultured in Cramer–Myers medium (CM) with 15 g/L glucose, 15 g/L fructose which is autoclaved

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separately to prevent Maillard reactions, 5 g/L Yeast extract (YE) and peptone each in replacement of  $(\text{NH}_4)_2\text{HPO}_4$ , Vitamin B12 0.5  $\mu\text{g/L}$  and Thiamine-HCl 100  $\mu\text{g/L}$ . The pH of the medium was set to 4.5 by the addition of 0.1N HCl. The heterotrophic cultivation in the bioreactor was carried out using an Eppendorf BioFlo 115 having Rushton type disk turbine impellers with pitched blade. The initial pH was kept at 4.5 and fermentation was done at uncontrolled pH. A working volume of 1.5 L was maintained and 10% inoculum was used for the initiation of the cultivation. The aeration rate, temperature and agitation were maintained at 1 vvm, 28 °C and 200 rpm respectively.

The *Chlamydomonas reinhardtii*, a known, non  $\beta$ -1,3-glucan producer was used as a control in prebiotic experiments and grown in Tris Acetate Phosphate medium at a pH of 7 (Kumari et al., 2020).

## 2.2. Culture conditions for *Lactobacillus*

*Lactobacillus*, Lacfid strain was obtained from Serum Institute of India, Pune. The Probiotic culture of *Lactobacillus* species was cultured in the MRS-broth (de Man et al., 1960) at 37 °C for 48 h with or without glucose at final pH of  $6.5 \pm 0.2$ . The glucose was autoclaved separately and added into the sterile medium to avoid charring.

## 2.3. Prebiotic study

*Lactobacillus* strain was grown with only MRS medium (control), or with MRS containing either autoclaved algae isolate (0.05 g/L) or autoclaved paramylon which was obtained from SDS purification (0.5 g/L dissolved in 0.5 N NaOH) in 500 ml flask with 200 ml working volume. Bacterial culture was incubated at 28 °C for up to 48 h in rotator shaker at 250 rpm. Samples were withdrawn in triplicates at different time points (0, 8, 16, 24, 32, 40, and 48 h). The sample was diluted as needed and plated onto complete MRS agar plates to determine viable bacterial count (cfu/ml). The control represents the *Lactobacillus* cells without the addition of *E. gracilis* or paramylon. Microalga *Chlamydomonas reinhardtii* which does not make  $\beta$ -1,3-glucan was used as the negative control. The standard paramylon was procured from Sigma Aldrich, USA and was used to compare the effect of SDS extracted paramylon.

## 2.4. Analysis

### 2.4.1. Biomass estimation

After centrifugation at 5000 rpm, biomass was washed twice with ddH<sub>2</sub>O. The pellet was dried at 70 °C for 48 h and dry biomass was obtained. Determination of the final biomass productivity of *E. gracilis* was done at the end of the log phase.

### 2.4.2. Paramylon extraction

Paramylon was isolated from *E. gracilis* cells by sodium dodecyl sulphate (SDS) and alkaline Extraction. For SDS extraction, paramylon was extracted and purified by resuspending 1 part of the cell into 26.5 part of a solution (g/g) containing 1 g/L sodium dodecyl sulphate and 50 g/L ethylene diamine tetra acetate dehydrate. This suspension was incubated for 60 min at 60 °C in the mini vortex at 1000 rpm and the paramylon granules were recovered by centrifugation for 10 min at  $6000 \times g$ . The SDS-Na<sub>2</sub>EDTA treatment was repeated and the paramylon was washed twice with ddH<sub>2</sub>O. After the second wash, the sample was dried overnight at 70 °C for weight determination. For the alkaline extraction, 100 mg of biomass was suspended into 30 ml methanol, incubated for 30 min at 50 °C with vortexing. This was followed by centrifugation for 10 min at  $6000 \times g$ . The pellet was washed twice with ddH<sub>2</sub>O. The pellet was suspended in 10 ml of 0.5N NaOH and incubated at 60 °C for 30 min. Paramylon was extracted by addition of Isopropyl alcohol followed by centrifugation for 10 min at  $6000 \times g$ .

### 2.4.3. Fourier transforms infrared spectroscopy

Fourier transform infrared spectroscopy (FT-IR) analysis was executed on a Shimadzu IR Prestige-21 instrument coupled to a Specac's

Golden Gate GS 10500 ATR assembly. Spectroscopy was performed in the ATR absorbance mode (range 4000–500  $\text{cm}^{-1}$  wave numbers with 20 scanning rate) at the resolution of 4  $\text{cm}^{-1}$ . The FTIR was done at transmission mode. The sample is pressed directly onto diamond crystal for ATR mode analysis. Analysis was done by IRsolution software package.

### 2.4.4. Scanning electron microscopy of paramylon

The scanning electron microscopy (SEM) (JEOL JSM 6380LA, Japan) of paramylon extracted from bioreactor grown *E. gracilis* cells was executed for study of morphological and surface features. Samples were dried prior to imaging and coated with double-sided adhesive tape and further coated with platinum in a sputter coater and examined at 15 kV at different magnifications. Images were captured at 2500 and 7000X magnification.

## 2.5. Statistical analysis

The experiment was performed in triplicates and all the values are presented as  $\pm$ SD. The P-value was calculated using mean, standard deviation and sample size with following equation:

$$s = \frac{\sqrt{(N_1 - 1)S_1^2 + (N_2 - 1)S_2^2}}{N_1 + N_2 - 2}$$

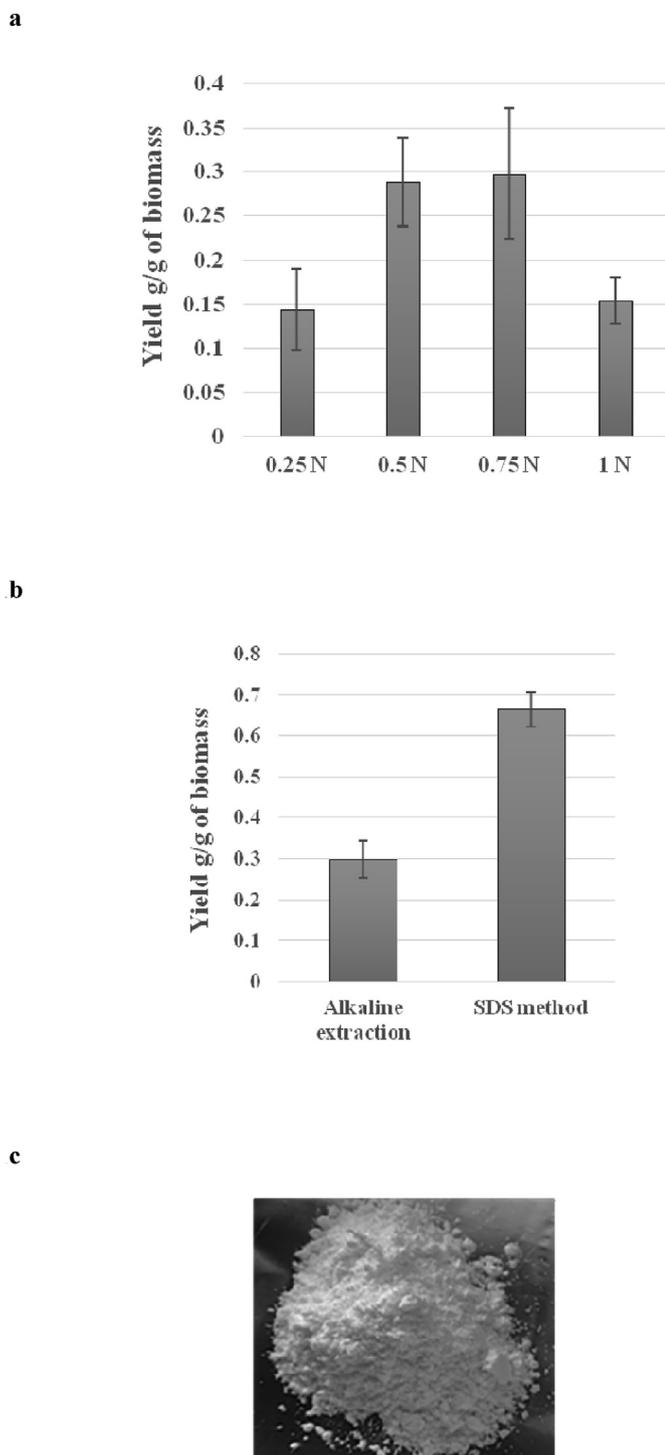
where S1 and S2 are the standard deviations of the two samples with sample sizes  $n_1$  and  $n_2$ .

P value was calculated using T-distribution for the two-tailed test.

## 3. Results and discussion

### 3.1. Cultivation of *E. gracilis* in heterotrophic mode for paramylon synthesis, isolation and characterization

*Euglena* has a broad spectrum substrate specificity and can utilize variety of pure sugars, sugar alcohols (Zhu and Wakisaka, 2020) as well as complex sugar and nitrogen containing waste sources (Rubiyatno et al., 2021) in both heterotrophic and mixotrophic mode. In the present work, pure food grade carbon and nitrogen sources were used for *Euglena* growth, since, the focus was on the evaluation of the prebiotic activity of *Euglena* cell powder and derived paramylon thereof. A biomass of 6.4 g/L with >50% paramylon content was obtained during heterotrophic growth of *E. gracilis* in media with glucose as carbon and YE and peptone as nitrogen source. Excess carbon (externally supplied in the medium) available to the *E. gracilis* cells gets stored typically as paramylon. The results are in accordance with the other reports (Wang et al., 2018). This heterotrophically grown biomass was used to isolate paramylon and its structure analysis and role as a functional dietary fibre was analysed. At 0.25 N NaOH concentrations, 0.14 g/g paramylon yield was obtained which was increased to ~ two fold at 0.5 N concentrations (Figure 1a). Further higher NaOH concentration did not lead to any substantial increase in the paramylon yield. The lower efficiency of alkaline extraction may be because of solubility constraints of the cells. The lower purity in alkali extracted paramylon can also be attributed to the solubility of the proteinaceous material in alkali along with paramylon (Tian et al., 2019). Paramylon is also known to dissolves into a kind of hydrogel when resuspended in alkaline environment (Matsumoto et al., 2021). This can be a reason for lower paramylon purification specifically at higher NaOH concentrations. SDS extraction was found to be more efficient as compared to that of alkali with 2.22-fold higher paramylon yield (Figure 1b). SDS extracted paramylon was white with uniform granular size, resembling closely with the analytical standard. SDS extraction method led to highest yield, purity and colour as illustrated in Figure 1c. The SDS extracted paramylon also matched identically upon FT-IR analysis (Figure 2) showing the characteristics peaks. SEM images (Figure 3) clearly depicted the intact paramylon isolation with SDS extraction while distortion in its structure by alkali method.



**Figure 1.** Extraction of paramylon (a) Effect of increasing concentration of NaOH on paramylon extraction (b) comparison of paramylon yield on biomass by alkali and SDS extraction (c) isolated paramylon from heterotrophically grown cells in the bioreactor.

### 3.2. FT-IR analysis of extracted paramylon

The aim of the study was to evaluate the prebiotic effect of the isolated paramylon from bioreactor grown *Euglena* cells and thus it was necessary to compare its purity with that to pure analytical grade. Vibration spectroscopic method like FT-IR can be used as purity control for the glucans like paramylon (Synytsya and Novak, 2014). In order to structurally characterize polysaccharides, two spectral regions “sugar

region” (1200-950  $\text{cm}^{-1}$ ) and “anomeric area” (950-750  $\text{cm}^{-1}$ ) are important. In the former region, the highly overlapping intense bands of CO and CC stretching vibrations in glycosidic bonds and pyranoid rings predominate. The latter region contains weak bands of complex skeletal vibrations sensitive to the anomeric structure of glucose. The FT-IR spectrum of SDS extracted paramylon matched closely with that of standard paramylon (Figure 2). Paramylon is a crystalline substance which exhibits abnormal dispersion, making the peak distorted. Figure 3 demonstrates the Kramers-Kronig Transform (to remove background noise from FT-IR data) to compare, SDS extracted paramylon with the standard one and shows close similarities.

The FT-IR spectrum of SDS extracted paramylon (Figure 2), shows the presence of 900  $\text{cm}^{-1}$  which is attributed to typical  $\beta$ -configurations i.e. (C1-H) deformation mode, and, therefore, indicates the presence of  $\beta$ -glucans. Broad peaks at 3300  $\text{cm}^{-1}$  indicate O-H stretch; while the signals at 2401 and 1215.72  $\text{cm}^{-1}$  indicate C-H stretch and  $\text{CH}_2\text{OH}$  stretch respectively. The two extra peaks which can be seen in SDS extracted paramylon is 661  $\text{cm}^{-1}$ , which is vibrations of C-C out of plane bending mode and 1421  $\text{cm}^{-1}$  is of CH bending deformation mode (Periathai and Rajagopal, 2014). The absence of peak between 1250-2000 ensured that isolated paramylon was free of protein (Synytsya and Novak, 2014).

### 3.3. Scanning electron micrography of SDS extracted paramylon from *E. gracilis*

Paramylon is known to be made in large quantities inside the euglenoid cell under abundant carbon concentrations and gets stored in the cytoplasm in the form of rod like bodies. The SEM showed the intact rod shaped paramylon granules from the heterotrophically grown *Euglena* cells (Figure 3a). The SEM analysis of the SDS extracted granule displayed intact ellipse shape with minor axis diameter of 2  $\mu\text{m}$  and major axis diameter of 3.5  $\mu\text{m}$  (Figure 3c). This was in accordance to the earlier reports where paramylon has been described and shown as micrometer-sized spheroidal particles (Monfils et al., 2011). The modification in paramylon structure is known to enhance the functional properties. There are reports of enhancement of immunomodulating activity by sonication and alkali treated paramylon (Kusmic et al., 2018). Paramylon is insoluble in water but gets dissolved in dilute NaOH (Kiss et al., 1988). In the present study, the paramylon was solubilized in NaOH for its application as prebiotic agent. Therefore, SEM was used to confirm the structural modifications induced by alkali treatment of isolated paramylon. The alkali treatment modified the paramylon structure demonstrating a major change in the surface topography as shown in Figure 3. NaOH treatment is reported to lead the reduction in degree of polymerization to  $\sim 70$  from  $\sim 3000$  and molecular weight to  $\sim 12$  kDa  $\sim$  from 500 kDa.

### 3.4. Prebiotic activity of *E. gracilis* cell extract and SDS extracted paramylon

Among algae a number of seaweeds and few microalgae are known to produce  $\beta$ -glucans. *Euglena* is a unicellular photosynthetic organism well known to produce crystalline insoluble  $\beta$ -1,3-glucan (Nakashima et al., 2018).  $\beta$ -glucans role in global food and pharmaceutical industry are increasingly being recognized.  $\beta$ -glucans are dietary fibres, present in the range of food products and are reported to have immunostimulatory, cholesterol lowering, antioxidative, prebiotic, anti-obesity and even antitumor activities (Nakashima et al., 2018). The beneficial health effects of  $\beta$ -glucans as prebiotics are contributed mostly by their influence on the composition of gut microbiota, growth and activities of probiotic bacteria like *lactic acid*, *bifidobacteria* etc (Davani et al., 2019). The prebiotic potential of soluble  $\beta$ -1,3-glucan from cereals as well as mushrooms are well documented (Vasiljevic et al., 2007; Ruthes et al., 2021) however long chain non-digestible paramylon prebiotic activities are largely unknown. Results of the *in vitro* growth study of *lactid* strain in the presence of 0.05% paramylon are shown in Figure 4. *Lactobacillus* growth

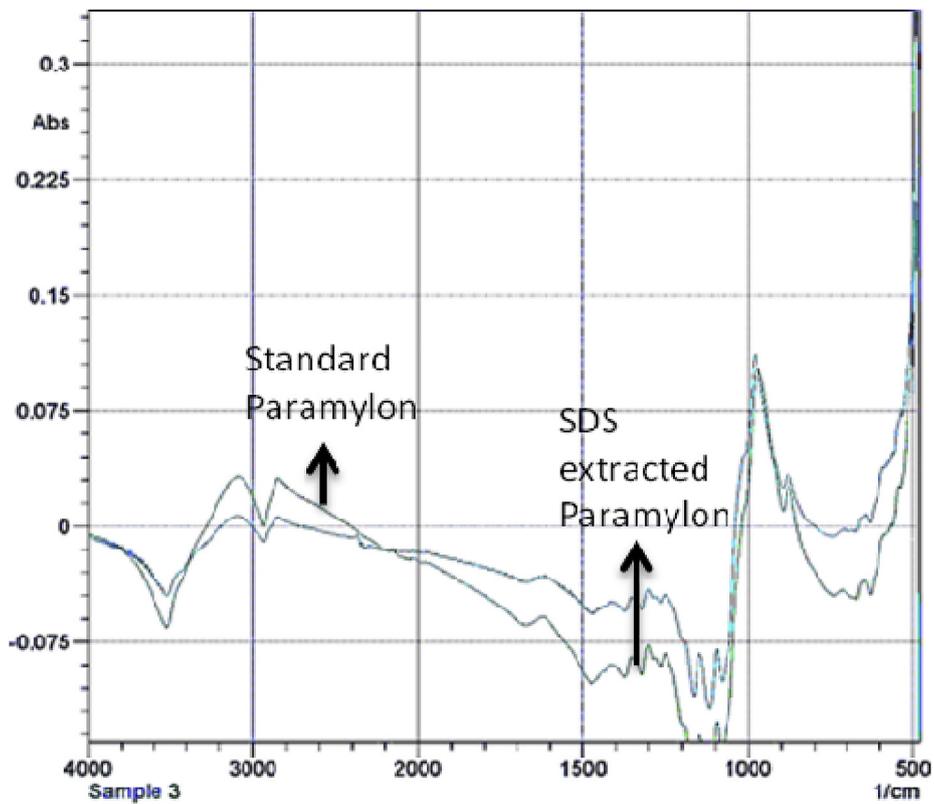


Figure 2. FT-IR analysis of SDS extracted paramylon with analytical standard.

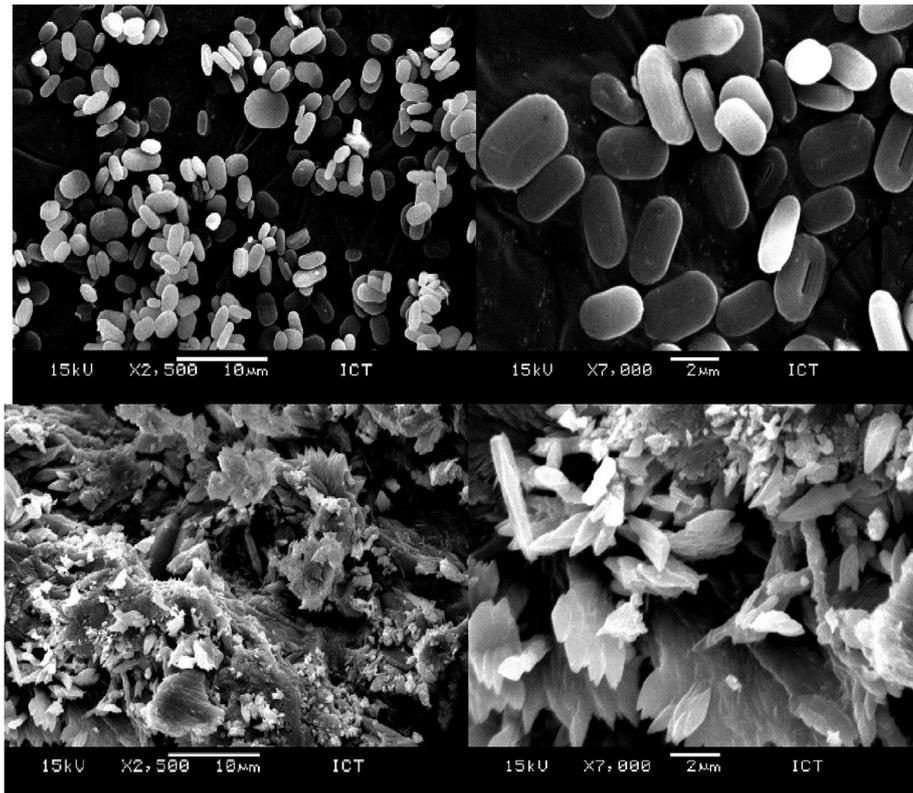
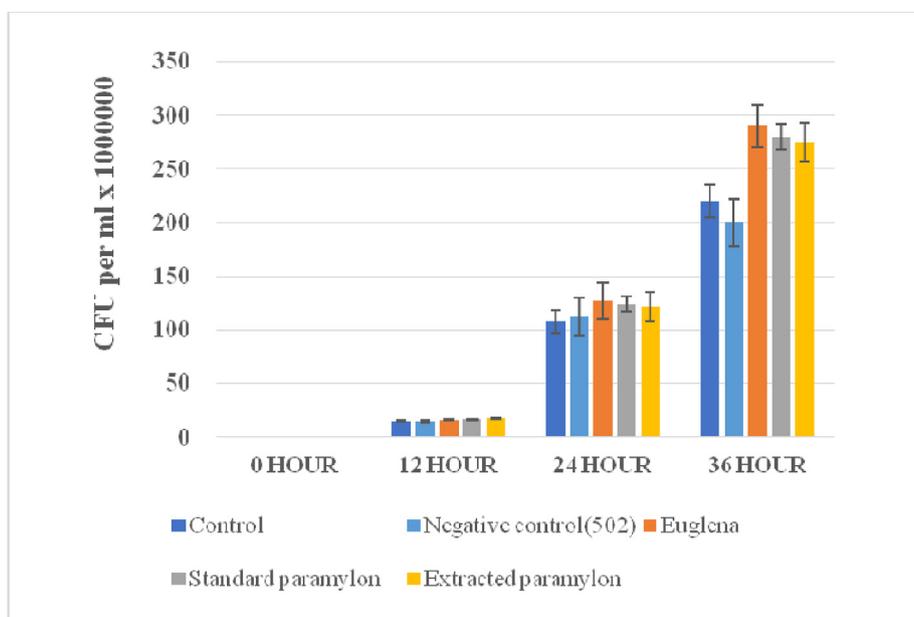


Figure 3. SEM of extracted paramylon; upper panel: SDS extracted: Left 2500X magnification, Right 7000X magnification; lower panel: Alkali extracted: Left 2500X magnification, Right 7000X magnification.



**Figure 4.** Comparison of prebiotic activity in terms of *Lactobacillus* growth in cfu/ml of control, negative control, standard and isolated paramylon, whole euglena cell powder.

remain unaffected despite the addition of algal powder (both *Chlamydomonas* and *Euglena*) or paramylon (analytical grade and purified) up to 24 h time duration (Figure 4). However at 36 h significant difference in *Lactobacillus* growth was obtained by addition of both *Euglena* powder and paramylon individually. Both analytical grade as well as *Euglena* biomass derived paramylon addition showed ~25% increase in cfu per ml of *Lactobacillus* as compared to that of control. The negative control i.e. *Chlamydomonas* cell lysate did not result in any significant increase of *Lactobacillus* even with prolonged incubation. *Chlamydomonas* does not contain  $\beta$ -glucans, therefore, prebiotic activity could predominantly be claimed to be associated with presence of  $\beta$ -1, 3-glucan either in pure form or enclosed inside the *Euglena* cells. It is noteworthy to note that Euglena.jp, a company claims increase in lactic acid bacteria in the presence of *Euglena* ([https://www.euglab.jp/en/column/euglena\\_cm/000463.html](https://www.euglab.jp/en/column/euglena_cm/000463.html)). It is not completely clear how whole *Euglena* cell powder was able to elicit the *Lactobacillus* growth. Lactic acid bacteria are known to be proteolytic (Emkani et al., 2021), responsible for protein aggregation (Berterame et al., 2016) and thus act as a permeabilizer and outer membrane-disintegrating agent (Alakomi et al., 2000) due to lactic acid production. Therefore, it can be hypothesized that *Euglena* which only has a proteinaceous pellicle membrane as outer covering might be more susceptible to leakage leading to paramylon and other cellular components release which might have aided in the *Lactobacillus* growth. Curdlan, another  $\beta$ -(1  $\rightarrow$  3)-d-glucan oligosaccharides have been reported to improve the growth of variety of *Lactobacillus* as well as *Bifidobacterium* species (Shi et al., 2018; Verma et al., 2020). Curdlan is also a linear  $\beta$ -1, 3-glucan similar to paramylon albeit it is soluble as opposed to that of later which is insoluble.

The paramylon itself is a recalcitrant substrate, however different pretreatment methods have been reported to obtain soluble  $\beta$ -1, 3-glucans (Gissibl et al., 2019; Kusmic et al., 2018). The paramylon treatment with sodium hydroxide is known to reduce the degree of polymerization (Guo et al., 2020) and also yield soluble nanofibers (Kataoka et al., 2002). These soluble nanofibers have been shown to enhance stimulation of leucocytes and hepatoprotection because of the formation of more bioactive single helices (Kusmic et al., 2018). In a recent study, alkali treated paramylon has been shown to activate the immuneresponse in murine RAW264.7 cells (Guo et al., 2020). The SEM of soluble paramylon clearly depicts the opening of the paramylon granules (Figure 3). Therefore, it can be postulated that bioactive single

nanofibre formed due to sodium hydroxide treatment of paramylon might have led to the prebiotic effect however; further investigation is required for better understanding of paramylon action as prebiotic.

#### 4. Conclusion

*Euglena gracilis* as whole cell and paramylon derived thereof has demonstrated the prebiotic effect on the *Lactobacillus*. *E. gracilis* is a protist with fast growth potential and high biomass generation in heterotrophic metabolism and also has a Generally Regarded as Safe (GRAS) status which is a prerequisite for any microbe for their application in the food industry. The enrichment of *Lactobacillus* derived products with *E. gracilis* or paramylon can have an additive effect on development of functional food with enhanced nutritive profile and thus hold great future potential.

#### Declarations

##### Author contribution statement

Tanmay Bhattad: Performed the experiments; Analyzed and interpreted the data.

Akshaykumar Koradiya: Performed the experiments.

Gunjan Prakash: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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##### Data availability statement

Data will be made available on request.

##### Declaration of interests statement

The authors declare no conflict of interest.

## Additional information

No additional information is available for this paper.

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